MICROORGANISMS IN SOLID MATERIALS

TASK I:

RESISTANCE OF ALPHA ORGANISMS TO DRYING AND TO STERILIZATION BY ETHYLENE OXIDE

(Mod. 1)

VOLUME III

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SUMMARY

Alpha has been identified as a variant of species Staphylococcus epidermidis. Organisms of this species are the most prevalent of microorganisms on all surfaces of human skin. They are also prevalent in the human mouth, nose, and respiratory tract. They are of low pathogenicity. The human being is not, however, the only source of Staphylococcus contamination.

Characteristically, organisms of the Staphylococcus species survive exposure to a wide variety of chemical agents and tolerate high concentrations of salts in liquid media. They are not resistant to heat as are bacterial spores. They appear to be sensitive to changes in their water content and the environment in which these changes are brought about. From some natural sources, populations of Staphylococcus organisms can be dried with preservation of most of the cells in a viable state. Drying from suspension in pure solvents destroys detectable viability in even large populations. Once the cells have been properly dried, however, they appear to remain viable for relatively long periods of time and to be resistant to the action of a variety of chemical agents.

Because of their agglomerating nature, quantitative assay of the number of viable cells in a population of Staphyloccus organisms is difficult.

I. INTRODUCTION

In assaying the internal microbial population of a specimen of solid propellant (Opfell, 1962 a and b) which had been prepared containing aluminum particles, liquid ethylene oxide, and an inoculum of spores of B. subtilis var. niger, the microorganism alpha was isolated. Because it survived in an environment which destroyed the detectable viability of the B. subtilis var. niger spores, alpha is of interest in demonstrating the effectiveness of processes for the preparation of sterile solid propellant.

Alpha and alpha-like species have been isolated subsequently from specimens of dust (Opfell, 1963a) from the JPL spacecraft assembly facility and from subsequent specimens of solid propellant (Opfell, 1964b).

The following paragraphs will describe and interpret the results of experiments to determine the ethylene-oxide resistance of populations of alpha in a variety of environments and after several types of pre-treatment. The evidence on which the classification Staphylococcus epidermidis has been assigned to this species will be presented and its significance in the engineering design of sterilization steps will be discussed.

The study described here represents a continuation of a recent study (Opfell, 1964a) to evaluate and to establish methods for assaying the viability of microbial populations dispersed in the interior of solid materials. A parallel study (Opfell, 1964b) and a previous study (Opfell, 1962a and b) applied these techniques to solid propellants. In both these studies populations of the species alpha or of species similar to it were found in the propellant specimens.

II. THE IDENTITY OF SPECIES ALPHA

On the basis of tests reported in Table I and comparison with the properties of ATCC strain 155 of Staphylococcus epidermidis, alpha has been identified to be a strain of Staphylococcus epidermidis. Though the distinction from species of Gaffkya is not as clear as one might like, the reasons for not calling alpha a species of Gaffkya are presented in Table II. As the discussion which follows will show, this distinction may not be really possible. Some bacteriologists (Stanier, 1963) call the staphylococci the pathogenic-members of the genus Micrococci and in particular use the term Micrococcus pyogenes, var. aureus for Staphylococcus aureus and Micrococcus pyogenes, var. albus for Staphylococcus albus.

Jones (1963) has studied fifteen strains of Staphylococcus epidermidis and found that some, like members of the genus Gaffkya, failed to reduce nitrates. One of the strains had a colored pigment. Jones listes the important characteristics of Staphylococcus epidermidis to be:

1) ability to grow anaerobically in a defined medium with glucose or pyruvate as energy source

2) inability to produce coagulase

3) ability to ferment serine

4) requirement for biotin and uracil under anaerobic conditions in a semisynthetic medium

5) inability to reduce nitrate beyond nitrite

Deibel (1960) proposed a set of characteristic properties for the genus Pediococcus. Adoption of this recommendation would eliminate the genus Gaffkya (U. S. Naval Medical School). The U. S. Naval Medical School (1963) points out the difficulty in distinguishing Gaffkya isolates from Staphylococcus epidermidis (coagulase negative). Gaffkya is distinguished by a high frequency of tetrads among cells isolated from

body fluids and by failure to reduce nitrate to nitrite. Many species of Micrococcus fail to reduce nitrate to nitrite (Skerman, 1959) and many including Staphylococcus epidermidis (also alpha) forms tetrads under several conditions of culture. Gaffkya can be easily distinguished from Staphylococcus aureus, however, by the nitrate and coagulase tests. Burrows (1954) assigns the name Micrococcus tetragena to Gaffkya tetragena, and shows a picture of a smear from pure culture. The picture would serve equally well as a description of alpha.

Skerman (1959) points out that considerable difficulty may be encountered in differentiating between the genus Micrococcus and the genera Staphylococcus and Pediococcus. He states that the genus Staphylococcus has been allocated only two species, Staphylococcus aureus and Staphylococcus epidermidis, omitting any mention of Staphylococcus albus. He suggests that the characteristics which differentiate the genus Pediococcus are based on byproducts produced in certain media and that comparable studies of the byproducts produced by the genus Staphylococcus and the genus Micrococcus have not been made.

Cowan (1964) suggests that catalase-positive Gram-positive cocci that ferment glucose under anaerobic conditions can be regarded as Staphylococci and that those which fail to do so are not Staphylococci -- they may be other species of Micrococci. He suggests also that Staphylococci be divided into two species: Staphylococcus aureus (coagulase-positive) and Staphylococcus epidermidis (coagulase-negative).

Bryan (1953) describes another species, Staphylococcus aerogenes, which is a strict anaerobe and might not apparently be a member of the genus in the terms defined by Cowan. Bryan says of Staphylococcus epidermidis that it is but Micrococcus epidermidis by another name and that it may be a less virulent variety of Staphylococcus albus. Frobisher (1962), on the other hand, equates the names Staphylococcus albus and Staphylococcus epidermidis.

III. THE HABITAT OF SPECIES ALPHA

The foregoing discussion shows the futility of inferring the range of habitat of alpha from the published literature on Staphylococcus epidermidis alone. Also, the confusion in the taxonomy of Staphylococcus epidermidis with that of similar species suggests that information on the habitats of other genera in the family Micrococcaceae or the tribe Streptococceae would be useful in estimating the significance of alpha in the environment surrounding the manufacture of sterile solid propellant. Among these genera are Micrococcus, Staphylococcus, Gaffkya, Sarcina, and perhaps even Pediococcus. Streptococceae organisms are found regularly in the mouth and intestinal tract of humans. In the mouth and respiratory tract Streptococceae cells are more prevalent (U. S. Naval Medical School, 1963) than are cells of the Micrococcaceae genera. On the exterior body surfaces, however, the Staphylococci are by far the most prevalent cells. Roseberg (1963) and Wilson (1955) point out that coagulase-negative staphylococci (Staphylococcus epidermidis) are prevalent on all areas of human skin. Roseberg also states that human skin normally carries a population of Clostridia and anaerobic cocci. Because growth of both Streptococceae populations and Clostridia populations is poor or absent on many of the media employed in the present tests, they might have been undetected in the propellant ingredients which were analyzed.

Gregory (1961) discusses reports of finding viable cells of Staphylococcus aureus and of Staphylococcus albus in the air over the open ocean. He suggests, however, that these populations came from land sources. The ability of cells of Staphylococcus species to survive in environments outside of living animals depends upon a variety of factors, many of which have not yet been explored.

An alpha population stored in purified water decays in a manner of multicellular organisms (Umbreit, 1962) which in a sense they are. A relatively small portion of the cells in a population of Staphylococcus cells occur singly. Most are in pairs, tetrads, or small clusters which do not break up easily.

IV. CLONAL VARIATION

Burrows (1954) points out that there is good reason to believe that bacterial populations are heterogenous with respect to the ability of the individual cells to withstand the effects of lethal environments. The implications of this hypothesis has been developed as a theory of clonal variation and selection. Heterogeniety is accounted for by the assumption that binary fission of a bacterial cell does not always produce daughter cells of precisely equal resistance to each of the lethal factors in the environment. The occurrence of two daughter cells of equal resistance is the most probable result of fission, of two daughter cells of slightly different resistance, slightly less probable, and of two daughter cells with much greater resistance, much less probable. Experience with the development of antibiotic resistance in strains of Staphylococcus aureus constitutes a large portion of the evidence in support of the theory of clonal variation.

When cultural conditions are changed, increasing numbers of the less resistant daughter cells may fail to reproduce with the result that over several generations, the average resistance of the cells in the culture has increased. With a return to the original cultural conditions, the resistance of the cells in the population will return to the original distribution of resistances after the passage of several generations. The populations of Staphylococcus epidermidis strains appear to be susceptible to this type of clonal variation. Staphylococcus epidermidis sp. x arose from the storage of a water suspension of alpha in the refrigerator for a year and a half.

V. ASSAY OF STAPHYLOCOCCUS EPIDERMIDIS

The variations in population growth rate among strains and even replicates of the same culture of a strain of Staphylococcus epidermidis is illustrated in Table III. The reason for the variations lies partially in the multicellular (Umbreit, 1962), form in which the viable particles of Staphylococcus epidermidis occur. Serial dilution breaks these particles apart and thereby extends the infectivity to greater dilutions through increasing number of independent viable particles. The greater the dilution, the greater the possibility for error in estimating the number of viable particles.

The grouping of the cells of Staphylococcus epidermidis has an effect on the ability of populations of them to withstand drying. The viable cells find themselves attached to colloidal particles (other cells, both living and dead for example). Umbreit (1962) points out that when bacteria are absorbed onto the surface of finely divided particles (colloid), their metabolic activity often slows down and they seem to survive much longer. After being so absorbed they are often resistant to drying. Protein materials also have a strong preservative effect during drying of many bacteria and viruses. No attempt was made to measure the effect of drying inoculums on the colloidal size particles of ammonium perchlorate (the principal ingredient in solid propellant) on the survival of cells in these inoculums. The water in the inoculum would dissolve ammonium perchlorate and on further drying the cells would be occluded in the ammonium perchlorate. The obvious test would not necessarily separate the colloidal effects from other effects of the ammonium perchlorate particles.

VI. RESISTANCE OF ALPHA POPULATIONS TO THE EFFECTS OF SEVERAL ENVIRONMENTAL FACTORS

On the surface of Trypticase soy agar at 37°C, alpha populations were found to remain viable for periods in excess of nine days. During this time the agar dried enough to become crisp. When alpha cells were collected, washed, and suspended in previously sterile purified water, the assay of viable cells did not change on storage of the suspension for periods up to a week. On eight-days' storage, however, the numbers of particles in the viable population appeared to drop by four to five orders of magnitude. After this drop in viability, the number of viable cells continued to fall but more slowly with some cells in the suspension remaining viable for very long periods of time. The viable population of Staphylococcus epidermidis ATCC 155 in water behaved similarly. No attempt was made to measure the ethylene-oxide or drying resistance of the survivors.

Table IV shows the comparative effects of suspension of viable cells of a species of Staphylococcus epidermidis and of spores of B. subtilis var. niger in several pure solvents. The toxicity of acetone for alpha populations was confirmed by the experiments reported in Tables VI, X, XI, and XII. Each of these three solvents are strong desiccants and likely withdraw water from the Staphylococcus cells in a manner other than optimum for preserving viability. That cells of Staphylococcus species are sensitive to their environment during desiccation is shown also by the experiment reported in Table IX and the studies reported by McDade (1963) and by Skaliy (1964). The presence of substances from agar or broth helped preserve viability in populations discussed in Table IX. McDade found that Staphylococcus aureus populations had variable resistance to drying under the synthetic conditions he used. His populations of about 1000 cells in broth were dried on sutures in an environment of air at several levels of relative humidity. Even at 50% RH, the condition most destructive of viability, his populations remained viable up to four days. Skaliy, on the other hand, found that one day was sufficient

for a Staphylococcus aureus population in a hospital room to be undetectable after the departure of a patient who had been confined there with a Staphylococcus aureus infection. Sykes (1958) reports that Staphylococcus aureus is the most resistant to drying of members of the Staphylococcus genus.

Using a cylinder-plate assay technique Snell (1954) measured the degree to which several organic solvents inhibit the growth of populations of several species of bacteria on an agar surface. Among the organic solvents tested were the three listed in Table III. Snell found one of the three to inhibit Micrococcus pyogenes, var albus (possibly, though not likely, the same strain as alpha) though both absolute ethanol and acetone inhibited Sarcina lutea, another species of the family Micrococcaceae. The conditions of exposure in Snell's study were substantially different from those used in the experiments corresponding to Table III. The desiccating effect of the solvent in Snell's cylinder-plate assay should have played a negligible role whereas it was possibly the dominant effect in the Table III experiment.

VII. TOXICITY OF PROPELLANT INGREDIENTS

The ability of a broth to support growth of a population of microorganisms in the presence of a toxic ingredient varies from batch to batch. In the tests reported in Table V, for example, an inoculum of 10^4 spores of B. subtilis var niger was able to grow in the presence of 0.5% v/v ethylene oxide. The three propellant ingredients, TDI, PPG, and AMP failed to suppress growth of any of the species tested even when the broth was saturated with them.

The toxicity of the propellant ingredients for alpha depends on the state of the alpha organisms at the time they are introduced into contact with the ingredient. In the tests reported in Table VI, the alpha organisms in 0.01 ml of purified water were placed in the bottom of previously sterile test tubes. The inoculum was permitted to dry, though not completely. (Some inoculums may have dried completely while others were seen to contain water at the time the propellant ingredients were placed on top of them). The exposure to ethylene oxide was made by placing the open tubes containing the inoculum and the propellant ingredient in a 4-mil polyethylene bag containing an atmosphere of air saturated with ethylene oxide vapor and water in excess of that equivalent to 50% RH. The tubes were exposed to this environment for 2 hours. The gas was flushed around the inside of the bag and over the tops of the tubes several times during the exposure period.

After removal from the bag the contents of each tube, including those not exposed to ethylene oxide, were stirred with a separate, sterile, glass stirring rod. The tubes were then stored at room temperature for the remainder of a 24-hour exposure period. After the end of this period, 5 ml of sterile fluid thioglycollate medium was put into each tube. Into each of the bacteriostasis control tubes were put 0.1 ml of purified water suspension of either alpha cells or spores of B. subtilis var niger containing approximately 100 viable particles each.

The tubes of broth were then incubated at 37°C for 7 days. A loopful of the broth was then streaked on the surface of Trypticase soy agar in a Petri dish. These streak plates were then incubated under the same conditions that the broths had been.

Generally, the propellant ingredients made the broths appear turbid so that microbial populations in the broth could not be detected on the basis of the appearance of the broth alone. The bacteriostasis tubes containing B. subtilis var. niger, however, contained an orange pellicle when the broth was not bacteriostatic.

In only one case, did the alpha population survive the sequence of conditions met in the test. This result is in contrast with the results shown in Table V for three of the ingredients. Moreover, the propellant ingredients appear to have made the broths bacteriostatic for inoculums of alpha containing but 100 viable particles in purified water. The inoculums used in the experiments corresponding to Table V included 10^4 viable particles.

The failure of the B. subtilis var. niger inoculums to grow in the broths containing Alrosperse (a reaction product of an equal mixture of stearic acid and diethynolamine) or to grow vigorously in those containing FeAA, unless these materials had been exposed to ethylene oxide is surprising and should be confirmed by further tests.

In view of the sensitivity of alpha to the conditions under which it is dried, the results shown in Table VI may not be surprising. Moreover, populations of 100 viable particles of Staphylococcus organisms seem to be more sensitive to toxic effects in broth than are populations greater than 10^4. This possibly is indicated in Table VIII.

VIII. NATIVE CONTAMINATION IN THE PROPELLANT INGREDIENTS

The broths at the "O" inoculum level would have disclosed native contamination in the propellant ingredients had there been a significant amount. The bacteriostasis controls show that these broths could have detected populations of 100 spores of organisms like B. subtilis var. niger but would fail to detect similar numbers of viable particles of alpha. This result does not mean that spores are necessarily more easily detected in the smaller numbers. Tables VII and VIII show that in broths containing ethylene oxide, larger populations of B. subtilis var. niger spores than of viable particles of Staphylococci were required to produce observable changes in the detection medium.

The low order of the contamination in several of the propellant ingredients was confirmed in the test made on duplicate samples of a Pfaudler-pot fuel mix (the results and the descriptions of the tests were reported by Opfell, 1964 b). This fuel mix contained Alrosperse, PPG and TMP.

The tests used were not sufficiently sensitive to support a conclusion that the propellant ingredients were sterile. The technique used to test them was not sterile, though care was taken to avoid accidental contamination.

IX. RESISTANCE OF SEVERAL SPECIES OF BACTERIA TO ETHYLENE OXIDE

The inoculum required to produce population growth in broth containing ethylene oxide depends upon the type of broth, the concentration of ethylene oxide and the species and strain of the microorgainsms. Tables VII and VIII compare the relative resistances of several species of microorganisms in both Trypticase soy broth and fluid thioglycollate medium and in the presence of a range of concentrations of ethylene oxide. In the 40 ml tubes closed with Morton closures, in which the broths were incubated, the concentration of ethylene oxide in the broth was confined to drop by no more than 50 percent on incubation at 37°C for 24 hours.

Ethylene oxide in the amount of 0.3% has been reported (Toplin, 1961) to sterilize fermentation broths. Tables VII and VIII show the levels of contamination by each of several species which might cause sterilization failure. Interestingly, the Staphylococcus strains are the most difficult (Sykes, 1958) to destroy under the conditions tested.

X. EFFECTS OF DRYING AND EXPOSURE TO ETHYLENE OXIDE ON THE VIABILITY OF STAPHYLOCOCCUS EPIDERMIDIS POPULATIONS

Populations of alpha cells and of the ATCC 155 strain of Staphylococcus epidermidis cells were compared with respect to their ability to withstand the effects of drying. The previously observed sensitivity of alpha populations to drying (Opfell, 1963 b) from suspension in purified water was used as a confirming test for alpha. The results reported in Table IX appear to support the conclusion that alpha is less resistant (Sykes, 1958) to drying than is the ATCC strain under identical conditions. Unfortunately, identical conditions are difficult to provide. The Tests 1 and 2 were performed on different days. The ATCC 155 strain cells used had been stored in purified water for a longer period of time than they had been for Test 1. The tests on the two strains were performed simultaneously, however.

The results of Tests 3 and 4 show the protective effects of nutrient substances during drying (Sykes, 1958). McDade (1963) found that populations of 1000 cells of Staphylococcus aureus dried on sutures from suspension in broth remained viable for four days and longer. Skaliy found that populations of Staphylococcus aureus from an infected patient died away in one day. Both these results appear to be consistent with the results presented in Table IX.

Alpha was isolated originally from the interior of a specimen of solid propellant. Presumably the cells got into the propellant originally accompanied by some type of protective substances. The environment inside the propellant was sufficiently protective to preserve viability for a long period of time.

Using a fractional factorial design, the relative effects of the several factors, surface supporting the inoculum during drying, the relative humidity and the oxygen content of the ambient gas during drying, the suspension vehicle, and exposure to ethylene oxide on the viability of

population of alpha were determined. Corresponding tests were performed on populations of spores of B. subtilis var. niger, which are known to survive the ethylene oxide exposure conditions used, to be unaffected by suspension in acetone, and to be insensitive to the methods of drying. The efficiency with which spores of B. subtilis var. niger can be removed from different types of surfaces is variable. Table XI presents the results of a control test on efficiency of recovery of the viable inoculum.

The details of the tests have been described in the footnotes to Table X. The concentrations of ethylene oxide and water vapor in the ambient gas during the exposure to ethylene oxide were confirmed by gas-chromatographic analysis.

The dominant factor in destroying viability of alpha populations was the drying process in the absence of protective agents. Table X shows quantitatively the frequency with which viable particles can be recovered from coupons on which viable cells had been dried. Though an attempt was made to remove the alpha inoculum from the surface of the coupon and suspend it in the broth, comparison of Table X with Tabes XI and XII shows that the treatment used was not entirely effective. Because of the possibility of accidental contamination of the coupons or of the broths in which they were incubated during the withdrawal of 0.5-ml aliquots from the jar, the recovery of viable alpha organisms from the coupons which had been inoculated with distilled water suspensions and had not been exposed to ethylene oxide was more than can be explained by contamination. After the effects of drying, the type of suspension vehicle is shown to be important but the type of solid material and the nature of the ambient gas did not produce discernable effects. The inoculum dried substantially more slowly on teflon than it did on the other materials, but this effect did affect the results.

TABLE I

CHARACTERISTICS OF STAPHYLOCOCCUS EPIDERMIDIS

Characteristics	"Bergey's Manual" Strain	Alpha
CELLS		
Shape	0.5-0.6 micron spheres	0.5 micron spheres
Gram-staining characteristics	Gram-positive	Gram-positive
Aggregation	singles, pairs, and irregular groups	singles, pairs, tetrads, and small groups
Mobility	nonmotile	nonmotile*
CULTURES		
Optimum temperature for growth	37°C	37°C
Oxidation-potential requirements	aerobic, facultative anaerobic	aerobic, facultative anaerobic
Nutritional factors required	amino acids for nitrogen, biotin, some B vitamins	appears to use ammonium from ammonium perchlorate
Salt tolerance	grows vigorously in media containing 10% sodium chloride	grows in 8% NaCl agar*
Appearance of broth containing fermentable carbohydrate	heavy uniform turbidity	medium uniform turbidity in TSB,** long thick "fingers" of turbidity in anaerobic part of FTM, gray-white sediment
Action on special media		
Litmus milk	acid	acid*
Nitrate broth	nitrate reduced to nitrite	nitrate reduced to nitrite
Glucose in broth	acid produced	acid produced*
Fructose in broth	acid produced	
Maltose in broth	acid produced	
Trehalose in broth	acid produced	
Sucrose in broth	acid produced	acid produced
Glycerol in broth	acid produced	
Mannose in broth	acid may be produced	

*Olitzky

**Trypticase soy broth and FTM indicates fluid thioglycollate medium

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TABLE I (Continued)

Characteristics	"Bergey's Manual" Strain	Alpha
CULTURES (Continued)		
Galactose in broth	acid may be produced	
Lactose in broth	acid may be produced	acid produced*
Xylose in broth	no acid produced	
Arabinose in broth	no acid produced	
Raffinose in broth	no acid produced	
Inulin in broth	no acid produced	
Sorbitol in broth	no acid produced	
Mannitol in broth	no acid produced	no acid produced*
COLONIES		
Gelatin-stab colonies	white surface growth with slow saccate liquifaction	slow liquifaction
Agar-surface colonies	smooth, circular, translucent white colonies	smooth, circular, translucent white colonies
OTHER CHARACTERISTICS		
Indole test		no indole produced*
Urease test		urease produced*
Catalase test	catalase positive	
Pathogenic	no	
Parasitic	yes	
Coagulase test	coagulase negative	
Hemolysis test		nonhemolytic

*Olitzky

TABLE I (Continued)

Characteristic	"Bergey's Manual" Strain	Alpha
ORIGINAL SOURCE	human skin wounds and abscesses	a specimen of solid propellant containing aluminum powder, ethylene oxide, and an inoculum of B. subtilis var. niger spores
HABITAT	skin and mucous membranes of humans and other animals	dust at JPL - possibly also people
DISTINCTIVE CHARACTERISTICS	ferments glucose but not mannitol under anaerobic conditions coagulase negative	appearance in FTM form of colonies frequency of tetrads in smears from surface colonies ethylene-oxide resistance sensitivity to drying from suspension in pure solvents

TABLE II

CHARACTERISTICS OF ALPHA COMPARED WITH THOSE OF GAFFKYA TETRAGENA

Characteristic	Gaffkya Tetragena+	Alpha
CELLS		
Shape	1 micron sphere	0.5 micron spheres
Gram-staining characteristics	Gram-positive	Gram-positive
Aggregation	singles, pairs, and tetrads (particularly in body fluids)	singles, pairs, tetrads, and small groups
Mobility	nonmotile	nonmotile*
Capsule	pseudocapsule in body fluids	
CULTURES		
Optimum temperature for growth	37°C	37°C
Oxidation-potential requirements	aerobic, facultative anaerobic	aerobic, facultative anaerobic
Nutritional factors required	amino acids for nitrogen biotin some B vitamins	appears to use ammonium from ammonium perchlorate
Salt tolerance		grows in 8% NaCl agar*
Appearance of broth containing fermentable carbohudrate	clear with gray sediment	medium uniform turbidity in TSB,** long thick "fingers" of turbidity in anaerobic part of FTM, gray-white sediment
Action on special media		
Litmus milk	acid	acid*
Nitrate broth	does not reduce nitrate*	nitrate reduced to nitrite
Glucose in broth	acid produced	acid produced*
Fructose in broth		
Maltose in broth		
Trehalose in broth		
Sucrose in broth		acid produced
Glycerol in broth	acid produced	
Mannose in broth		

*Olitzky

**Trypticase soy broth and FTM indicates fluid thioglycollate medium

+ Bryan

TABLE II (Continued)

Characteristic	Gaffkya Tetragena	Alpha
CULTURES (Continued)		
Galactose in broth		
Lactose in broth	acid produced	acid produced
Xylose in broth		
Arabinose in broth		
Raffinose in broth		
Inulin in broth		
Sorbitol in broth		
Mannitol in broth		no acid produced
COLONIES		
Gelatin-stab colonies	white surface growth but with no liquification	
Agar-surface colonies	smooth, circular, translucent white, glistening colonies with entire edge	smooth, circular, translucent white colonies
OTHER CHARACTERISTICS		
Indole test		no indole produced
Urease test		urease produced
Catalase test		
Pathogenic	no	
Parasitic		
Coagulase test	coagulase negative	
Homolysis test		nonhemolytic
Gas production	none+	none

+ Bryan
* U. S. Naval Medical School

TABLE II (Continued)

Characteristic	Gaffkya Tetragena	Alpha
ORIGINAL SOURCE	human skin wounds and abscesses	a specimen of solid propellant containing aluminum powder, ethylene oxide, and an inoculum of B. subtilis var. niger spores
HABITAT	skin and mucous membranes of humans and other animals, and in the air	dust at JPL - possibly also people
DISTINCTIVE CHARACTERISTICS	tetrads in body fluids does not reduce nitrates growth as sediment in broth	appearance in FTM form of colonies frequency of tetrads in smears from surface colonies ethylene oxide-resistance sensitivity to drying from suspension in pure solvents

TABLE III

GROWTH RATE OF STAPHYLOCOCCUS EPIDERMIDIS STRAINS

Tubes of Trypticase soy broth which showed turbidity after inoculation with two different strains of Staphylococcus epidermidis and incubation at 37°C for several periods of time

Length of Incubation hours	Strain[1]	Size of Inoculum[2], logarithm of one-half the estimated number of viable particles											
		6	5	4	3	2	1	0	-1	-2	-3	-4	-5
16	x	+	+	-	-	-	-	-	-	-	-		
	155a			+	+	-	-	-	-	-	-	-	-
	155b			+	+	-	-	-	-	-	-	-	-
19	x	+	+	+	+	+	-	-	-	-	-		
	155a			+	+	+	-	-	-	-	-	-	-
	155b			+	+	+	-	-	-	-	-	-	-
24	x	+	+	+	+	+	+	-	-	-	-		
	155a			+	+	+	+	+	-	-	-	-	-
	155b			+	+	+	+	-	-	-	-	-	-
40	x	+	+	+	+	+	+	-	+	-	+		
	155a			+	+	+	+	+	+	+	-	-	-
	155b			+	+	+	+	+	-	-	-	-	-
48	x	+	+	+	+	+	+	-	+	-	+		
	155a			+	+	+	+	+	+	+	-	-	-
	155b			+	+	+	+	+	-	-	-	-	-

1. Strain x is a mutant of species alpha which is not resistant to ethylene oxide dissolved in broth to the extent of 0.4% and which does not liquify gelatin as alpha does. Strains 155a and 155b are replicates, of a suspension of cells in purified water, obtained from a single ampoule of ATCC 155 strain of Staphylococcus epidermidis.

2. Cells of Staphylococci occur mostly in multiples, i.e., diploids, tetrads, and small clusters (the name means cocci which occurs in grapelike clusters). For this reason, assays disclose the numbers of viable clusters or particles. On serial dilution, some of the clusters are broken into smaller clusters which extends the range of infectivity of suspensions to greater dilutions. It also means that at the higher concentrations the actual number of viable clusters is less than that indicated by conventional interpretations of serial dilution assays.

TABLE IV

TOXICITY OF SOLVENTS FOR MICROORGANISMS

Survival of microorganism populations (2×10^4 cells) in suspension in several common solvents at room temperature for two periods of time.

Suspension Vehicle	B. subtilis. var. niger		Staph. epidermidis, sp.	
	0 hours	4 hours	0 hours	4 hours
Water	+	+	+	+
Ethanol 99%, water 1%	+	+	-	-
Acetone 99%, water 1%	+	+	-	-
Methanol 99%, water 1%	+	+	-	-

1. One hundred thousand (10^5) cells in 0.1 ml of water were dispersed in 10 ml of the solvent. Five percent of this population, in 0.5 ml of the solvent, was transferred to Trypticase soy agar and incubated at 37°C for 3 days. The + signs indicate some of the population survived. The - signs indicate that no survivors were detected.

TABLE V

TOXICITY OF PROPELLANT INGREDIENTS

+ indicates the inoculum grew in the broth, - indicates that it did not grow.

Species	Broth	Propellant Ingredient																					
		Log_{10} of % V/V ETO[1]						Log_{10} of % V/V TDI					Log_{10} of % V/V PPG						Log_{10} of % W/V AMP				
		1.2	0.5	-0.3	-1.0	-2.7	-∞	1.2[3]	0.5[3]	-0.3[3]	-1.0[3]	-2.7[3]	1.2[3]	0.5[3]	-0.3	-1.0	-2.7	-∞	1.5[3]	0.7	-0.1	-0.8	-1.5
B. subtilis, var. niger spores	TSB[2]	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	FTM	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Staph. epidermidis sp. x	TSB	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	FTM	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Staph. epidermidis ATCC 155	TSM	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	FTM	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

1. ETO is ethylene oxide. The concentration is expressed in terms of volume of liquid ethylene oxide
   TDI is 2,4-toluene diisocyanate
   PPG is polypropylene glycol
   AMP is ammonium perchlorate
   TSB is Trypticase soy broth
   FTM is fluid thioglycollate medium

2. Into each tube containing 5 ml of the indicated broth were put 1 ml of the propellant ingredient, suspended in enough water to produce the proper concentration, and 0.1 ml of a water suspension of the microorganisms containing 10^4 viable particles.

3. The broth was saturated with the propellant ingredient (in the case of TDI, the reaction product of TDI and water). A second phase, consisting primarily of the propellant ingredient or reaction product, was also present in the broth tube.

TABLE VI

TOXICITY OF PROPELLANT INGREDIENTS

Vigor of Microbial Growth in Broth

Ingredient	Inoculum	Exposed to Ethylene Oxide	No	No	Yes	Yes
		Exposed to Ammonium Perchlorate	No	Yes	No	Yes
Aluminum Powder (MD 105, Lot # A1163-112)	10^6		+			
	10^4					
	10^2					
	0					
	10^2A^2				+	
	10^2B^3		++		++	
Alrosperse (Lot # 85377)	10^6					
	10^4					
	10^2					
	0					
	10^2A^2					
	10^2B^3				++	
PBNA, Phenyl-beta-naphthylamine (Lot #54)	10^6					
	10^4					
	10^2					
	0					
	10^2A^2					
	10^2B^3		++		++	++
TMP, 1, 1, 1-Trimethylol propane	10^6					
	10^4					
	10^2					
	0					
	10^2A^2					
	10^2B^3		++	Δ+	++	++
FeAA, Ferric acetylacetonate (xx mod., 0.010 screen, ground 8-3-63	10^6					
	10^4					
	10^2					
	0					
	10^2A^2					
	10^2B^3		+		++	
Acetone	10^6					
	10^4					
	10^2					
	0					
	10^2A^2					
	10^2B^3		++		++	
TDI, 2, 4-Toluene diisocyanate (Lot #279)	10^6					
	10^4					
	10^2					
	0					
	10^2A^2					
	10^2B^3		++		++	
PPG, Polypropylene glycol (BH-3-252278 DR 21 degassed 2-2-64)	10^6					
	10^4					
	10^2					
	0					
	10^2A^2					
	10^2B^3		++		++	++

[1] (Lot #7883 DR 27 and 28)

[2] Bacteriostasis control for Alpha

[3] Bacteriostasis control for B. subtilis, var. niger spores

[4] While B. subtilis, var. niger did not grow, a contaminant did.

TABLE VII

ETHYLENE OXIDE RESISTANCE OF SEVERAL SPECIES OF BACTERIA

+ indicates that the inoculum grew in the broth when incubated 40 hours at 37°C, - indicates that the inoculum did not grow.

Species	Inoculum, particles[3]	% V/V of Liquid Ethylene Oxide[1] in TSB[2]				
		0.60	0.40	0.20	0.10	0.5 or less
B. subtilis, var. niger spores	10^2	-	-	-	-	+
		-	-	-	-	-
	10^6	-	-	+	+	+
		-	-	+	+	
Staph. epidermidis ATCC 155	10^4	-	-	-	+	+
		-	-	-	+	+
	10^5	-	-	+	+	+
		-	-	+	+	
Staph. epidermidis sp. x	10^4	-	-	-	+	+
		-	-	+	+	+
Alpha	10^4	-	-	-	+	+
		-	+	+	+	+
	10^5	+	+	+	+	+
		-	+	+	+	
		-	+	+	+	
		-	-	+	+	

1. The ethylene oxide was contained in 1 ml of an aqueous solution.
2. TSB means Trypticase soy broth.
3. The inoculum was contained in 0.1 ml of viable particles suspended in water.

TABLE VIII

INOCULUMS OF EACH OF SEVERAL SPECIES OF MICROORGANISMS REQUIRED TO INFECT FLUID THIOGLYCOLLATE MEDIUM CONTAINING ETHYLENE OXIDE
Number of + signs indicates density of turbidity. - signs indicate no turbidity appeared.

Species	Inoculum	Percent of Ethylene Oxide in Fluid Thioglycollate Medium			
	Cells	0.00	0.38	0.70	0.96
Bacillus from ethylene oxide-exposed SY 251/7 propellant specimen, species Zavin from Task II.	3×10^7	---	---	---	---
	3×10^6	+++	+++	+++	+++
	3×10^5	+++	+++	++	++
	3×10^4	+++	+++	++	++
	3×10^3	+++	+++	-	-
	3×10^2	+++	+++	++	-
B. lichenformis	1×10^7	---	---	---	---
	1×10^6	+++	++	++	-
	1×10^5	+++	++	-	-
	1×10^4	+++	++	-	-
	1×10^3	+++	-	-	-
	1×10^2	---	---	---	---
Beta	1×10^7	---	---	---	---
	1×10^6	+++	+++	+++	-
	1×10^5	+++	+++	+++	+++
	1×10^4	+++	+++	+++	+++
	1×10^3	+++	+++	-	-
	1×10^2	+++	-	-	-
B. subtilis, var. niger	3×10^7	+++	+++	-	-
	3×10^6	+++	+++	-	-
	3×10^5	+++	++	-	-
	3×10^4	+++	++	-	-
	3×10^3	+++	-	-	-
	3×10^2	---	---	---	---
Alpha	2×10^7	+++	+++	+++	+++
	2×10^6	+++	+++	+++	+++
	2×10^5	+++	+++	+++	+++
	2×10^4	+++	+++	+++	+++
	2×10^3	+++	+++	+++	++
	2×10^2	---	---	---	---
Staphylococcus epidermidis	1×10^7	---	---	---	---
	1×10^6	+++	++	+	+
	1×10^5	+++	++	++	+
	1×10^4	+++	++	+	+
	1×10^3	+++	++	+	+
	1×10^2	+++	++	+	-

TABLE IX

EFFECT OF DRYING ON VIABILITY OF
STAPHYLOCOCCUS EPIDERMIDIS POPULATIONS

Numbers of colonies on Trypticase soy agar incubated at 37°C

Inoculum[1]	Expected Number of Colonies	Strain						
		ATCC 155		Alpha				
		Test 1	Test 2	Test 1	Test 2	Test 3	Test 4	Test 5
10^8	2,000,000				320			
10^7	200,000		496	4	50			TNC
10^6	20,000	1000	7	1	1	TNC	126	
10^5	2,000	260	7	0	0	20	80	
10^4	200	0	0	0	0	1	4	
10^3	20	0		0		0	0	

1. The inoculum was contained in 0.1 ml of a liquid suspension of the cells. It was dried on a glass cover slip in an open Petri dish for a period of 4 hours. The inoculum remained saturated for two of these four hours. In both Tests 1 and 2 for both Strains, the cells had been washed before they were suspended in sterile purified water and used to inoculate the glass cover slips. In Tests 1 and 2 the inoculum was dried in air at 50% RH. The cells used in Tests 3, 4, and 5 had not been washed. For Tests 3 and 4 the inoculums represent a serial dilution of material flushed from a partially dried culture on the surface of Trypticase soy agar. Though the dilutions were made in sterile purified water, some of the agar and nutrients surely accompanied the cells into the inoculum where they could protect the cells from destruction by drying. In Test 5 the inoculum was from a three-day-old culture in fluid thioglycollate medium. While the inoculum in Test 5 was dried simultaneously with and similarly to that of Test 2, the inoculum for Test 3 was dried in dry air and for Test 4 in dry nitrogen.

TABLE X

EFFECTS OF DRYING AND EXPOSURE OF ETHYLENE OXIDE ON POPULATIONS OF ALPHA ORGANISMS

Number[1] of colonies on each Trypticase soy agar plate after inoculation with a 0.5-ml aliquot of the fluid thioglycollate suspension[2] of the dried[3] inoculum.

Support	Desiccant	Replicate	Exposed[4] to Ethylene Oxide Vapor				Not Exposed to Ethylene Oxide Vapor			
			In Acetone		In Water		In Acetone		In Water	
			Alpha	Spores	Alpha	Spores	Alpha	Spores	Alpha	Spores
Glass Coupon, 1"x 1" x 0.05"	Dry Nitrogen	1		0				5		60
		2		0				0		100
	Dry Air	1	0			0		6	0	
		2	0			0		0	0	
	Air at 50% RH	1	0			0		10	0	
		2	0			0		42	0	
Teflon Coupon, 1" x 1"x 0.01"	Dry Nitrogen	1	0			0		0	0	
		2	0			0		0	0	
	Dry Air	1	0			0		1	0	
		2	0			1		1	0	
	Air at 50% RH	1		0	0		0			62
		2		2	0		0			70
Aluminum Coupon, 1" x 0.03"	Dry Nitrogen	1	0					19	0	
		2	0					24	0	
	Dry Air	1		0	0		0			28
		2		2	0		0			25
	Air at 50% RH	1	0					15	0	
		2	0					5	0	

1. Expected number of colonies, had all viable particles in the inoculum on the coupon survived drying and had been evenly dispersed in the fluid thioglycollate medium, was 2×10^4. The inoculum on the coupon contained, initially, 10^6 particles in 0.1 ml of liquid. On drying these particles could form clumps which were not dispersed adequately enough to disclose the true numbers of survivors.
2. The dried inoculum was suspended in previously sterile fluid thioglycollate medium by putting the coupon (glass, teflon, or aluminum) into the medium aseptically and then sonicating the medium for 1 minute before shaking the jar of medium and withdrawing the 0.5-ml aliquot. The coupons were all prepared and tested in duplicate.
3. The inoculum included 10^6 viable particles (alpha occurs mostly as diploids, tetrads, and small groups of cells) dried for 24 hours in the indicated environments.
4. The exposure was to saturated ethylene oxide vapor, containing water vapor in excess of that equivalent to 50% RH and a small amount of air. The exposure took place at room temperature. Exposure lasted for 2 hours.

TABLE XI

EFFECT OF DRYING AND EXPOSURE TO ETHYLENE OXIDE ON DETECTABILITY OF POPULATIONS OF ALPHA ORGANISMS[1]

The number of + signs indicates the turbidity of the fluid thioglycollate medium containing the dried inoculum and coupon after incubation, - signs indicate that no turbidity appeared.

Support	Desiccant	Replicate	Exposed to Ethylene Oxide Vapor				Not Exposed to Ethylene Oxide Vapor			
			In Acetone		In Water		In Acetone		In Water	
			Alpha	Spores	Alpha	Spores	Alpha	Spores	Alpha	Spore
Glass	Dry Nitrogen	1		-	++			+++		+++
		2		-	-			+		+++
	Dry Air	1	+++			-		+++	-	
		2	+++			-		+++	+++	
	Air at 50% RH	1	-			++		+++	-	
		2	-			-		+++	+++	
Teflon	Dry Nitrogen	1	-			+		+++	+	
		2	-			+++		+++	+	
	Dry Air	1	-			+++		-	+	
		2	-			-		+++	+	
	Air at 50% RH	1		-	-	-	-			+++
		2		++	-	-	+			+++
Aluminum	Dry Nitrogen	1	-					+++	-	
		2	-					+++	+++	
	Dry Air	1		-	-		+			+++
		2		-	-		+			+++
	Air at 50% RH	1	+++			-		+++	+	
		2	-			-		+++	+	

1. The populations and their treatments are described in the footnotes to Table X.

TABLE XII

COLOR[1] OF FLUID THIOGLYCOLLATE MEDIUM CONTAINING DRIED INOCULUM AND COUPON AFTER INCUBATION[2]

Support	Desiccant	Replicate	Exposed to Ethylene Oxide Vapor				Not Exposed to Ethylene Oxide Vapor			
			In Acetone		In Water		In Acetone		In Water	
			Alpha	Spores	Alpha	Spores	Alpha	Spores	Alpha	Spores
Glass	Dry Nitrogen	1		straw	yellow			yellow		yellow
		2		straw	amber			amber		yellow
	Dry Air	1	yellow			straw		yellow	amber	
		2	yellow			straw		yellow	amber	
	Air at 50% RH	1	amber			amber		yellow	amber	
		2	amber			amber		yellow	amber	
Teflon	Dry Nitrogen	1	straw			amber		yellow	amber	
		2	straw			yellow		straw	amber	
	Dry Air	1	straw			yellow		yellow	amber	
		2	straw			straw		yellow	amber	
	Air at 50% RH	1		amber	amber		amber			yellow
		2		amber	amber		amber			yellow
Aluminum	Dry Nitrogen	1	yellow					yellow	amber	
		2	straw					yellow	amber	
	Dry Air	1		straw	straw		amber			yellow
		2		straw	straw		amber			yellow
	Air at 50% RH	1	straw			amber		yellow	amber	
		2	straw			amber		yellow	amber	

1. A 20-ml quantity of freshly prepared fluid thioglycollate medium in baby-food jars is straw colored. Because the head space over the medium contains oxygen, the broth soon becomes saturated and amber in color, but remains free of turbidity. Characteristically, the medium becomes increasingly reducing as populations of microoganism grow and consume oxygen. The amber color than disappears and a yellow color develops. The yellow color is usually accompanied by a dense turbidity. Occasionally the microorganism population does not become large enough to produce turbidity but the broth develops a straw color again, indicating oxygen consumption.

2. The broth had been sampled to prepare the agar plates described in Table X. Moreover, some coupons contained viable particles of species other than alpha or B. subtilis, var. niger. These particles produced yellow color in the broth. In interpreting the table, "amber" means that the broth appeared to contain no viable nor metabolizing cells from the inoculum. All amber colored broths were confirmed to be nonbacteriostatic.

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